# Preliminary investigations on the chemical composition of the scale-boundary and cyst wall of *Pyramimonas pseudoparkeae* (Prasinophyceae)

M.E. Aken and R.N. Pienaar

Department of Botany, University of Natal, Pietermaritzburg

Scales of *Pyramimonas pseudoparkeae* Pienaar *et* Aken were isolated by differential centrifugation and treated with cellulase, pectinase, HCl, NaOH, EDTA, boiling water and alcian blue (pH 2,5 and pH 0,5). The component sugars and amino acids in the scales were detected in acid hydrolysates using TLC and an amino acid analyser. Scales are predominantly carbohydrate in composition and contain approximately 4% protein. Scale polysaccharide is pectinaceous in nature, containing polygalacturonic acid and neutral sugars, galactose, arabinose, xylose, rhamnose and (trace) fructose. The scale polysaccharide is sulphated. Aspartic and glutamic acid represent major amino acid residues in scale hydrolysates. The polyanionic nature of the scales may explain the external self-assembly of the scale-boundary. The scale-boundary may also contribute to the maintenance of the water and salt balance in the cell. Histochemical tests on the bilayered cyst wall revealed that the outer component, which withstood acetolysis, is probably composed of sporopollenin. The inner layer has the same staining properties as the scales and is probably similar in composition. This layer also contains a sulphated acidic polysaccharide and some lipoidal material. The cyst wall is PAS-negative.

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Skubbe van *Pyramimonas pseudoparkeae* Pienaar *et* Aken is met behulp van gedifferensieerde sentrifugering geïsoleer en met sellulase, pektinase, HCl, NaOH, EDTA, kokende water en alsien blou (pH 2,5 en pH 0,5) behandel. Die suikers en aminosure in die suurhidrolisate van skubbe is bepaal deur gebruik te maak van dunlaag-chromatografie en 'n aminosuuranaliseerder. Die skubbe bestaan hoofsaaklik uit koolhidrate en bevat ongeveer 4% proteïen. Die sulfaatbevattende skub-polisaggariede is pektienagtig en bevat poligalakturoonsuur en neutrale suikers soos galaktose, arabinose, xilose, ramnose en klein hoeveelhede fruktose. Aspartien- en glutamiensuur is die belangrikste aminosuurreste in skub-hidrolisate. Die poli-anioniese samestelling van die skubbe mag 'n verklaring bied vir die eksterne self-saamvoeging van die skub-grens. Die skub-grens mag ook bydra tot die handhawing van die water en soutbalans in die sel. Histochemiese toetse op die tweelagige sistwand het aan die lig gebring dat die buitenste laag, wat asetolise weerstaan, waarskynlik saamgestel is uit sporopollenien. Die binneste laag het dieselfde kleuringseienskappe as die skubbe en is waarskynlik identies in samestelling. Hierdie laag bevat ook 'n sulfaatbevattende suur-polisaggaried, asook lipoïede-materiaal. Die sistwand is PAS-negatief.

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**Keywords:** Acidic polysaccharide, cyst wall, *Pyramimonas*, scale composition, sporopollenin



**M.E. Aken and R.N. Pienaar***

Department of Botany, University of Natal, P.O. Box 375, Pietermaritzburg, 3200 Republic of South Africa

*To whom correspondence should be addressed

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## Introduction

*Pyramimonas pseudoparkeae* Pienaar *et* Aken (Pienaar & Aken 1985) is a unicellular, quadriflagellate green monad belonging to the class Prasinophyceae (Norris 1980). The alga is found in tidal pools on the south, east and west coasts of South Africa.

Flagellate cells of members assigned to the class possess a covering of scales on the flagellar and often on the cell surfaces. Prasinophytes are thought to be extant representatives of a group of primitive green algae that gave rise to all other chlorophytan algae and ultimately the land plants (Moestrup 1974; Pickett-Heaps & Ott 1974; Stewart & Mattox 1978; Domozych *et al.* 1980; Norris 1980; Melkonian 1982a).

*P. pseudoparkeae*, like all other species within the genus, possesses a complex covering of unmineralized organic scales (Pienaar & Aken 1985). The cell body is covered with three different scale types which are arranged in superimposed layers. The flagella bear four scale types.

Scales are synthesized throughout the cell division cycle by two anteriorly situated dictyosomes (Aken & Pienaar 1981). All scale types may be synthesized simultaneously within a single dictyosomal cisterna. The flagellar and body scales constitute what will be called the scale-boundary. The term periplast is used to refer to the body scale covering only.

While much is known about scale morphogenesis within the genus (Manton 1966; Moestrup & Walne 1979), nothing is known about the chemical composition of the scales. The periplast has sometimes been proposed as the possible precursor to the cell wall of chlorophycean green algae (Mattox & Stewart 1977; Domozych *et al.* 1980; Norris 1980) but there is as yet no chemical evidence to support this.

Many workers have drawn attention to the need to investigate the chemical composition of prasinophyte scales but to date no such studies have been reported (Moestrup & Walne 1979; Domozych *et al.* 1980; Melkonian & Robenek 1981; Melkonian *et al.* 1981; Norris 1982). Lewin (1958), however, has analysed the theca of the prasinophyte *Tetraselmis* Stein and has shown that it is predominantly carbohydrate in composition with thecal hydrolysates yielding galactose, uronic acid and traces of arabinose. Gooday (1971) confirmed these observations by demonstrating that the theca is pectin-like with galactose, galacturonic acid, arabinose and rhamnose representing the major constituents. A later study by Manton and co-workers revealed that the theca contains significant amounts of calcium, probably present as the calcium salt of galacturonic acid (Manton *et al.* 1973). The theca is composed of small coalesced scale-like particles (Manton & Parke 1965) and may be homologous with the periplast of *Pyramimonas*

Schmarda.

In ageing cultures, *P. pseudoparkeae* enters an asexual reproductive cycle (Aken in prep.) in which flagellate cells containing large quantities of storage products lose their flagella, become spherical, and deposit a thick bilayered cyst wall beneath the periplast.

The purpose of this article is to present current data on our efforts to determine the chemical composition of the scales and cyst wall of *P. pseudoparkeae*.

## Materials and Methods

*P. pseudoparkeae* was isolated (RNP) from a tidal pool sample collected at Oudekraal on the Cape Peninsula in 1976. The alga is maintained in unialgal clonal culture in Provasoli's enriched sea-water medium (Provasoli 1968) at 20°C in a 16L:8D photoregime (light intensity 150 $\mu Em^{-2}s^{-1}$). Mass cultures of the alga were obtained by culturing cells in 10 $\ell$ of medium in glass carboys. Cultures were incubated under the conditions described above and were aerated with 2% $CO_2$ in air (flow rate 2 $\ell$ $min^{-1}$).

### Isolation of scale fractions

Cells were harvested after 14 days (cell density $10^5$ cells $ml^{-1}$) and two scale fractions were collected by differential centrifugation as outlined in Figure 1. No attempt was made to

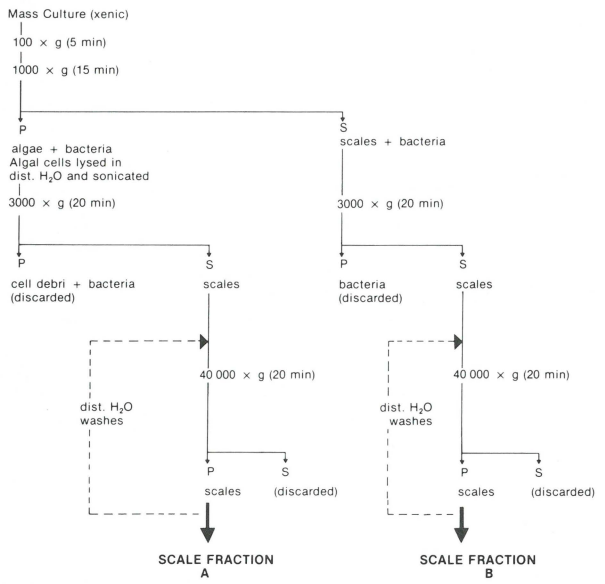



**Figure 1** Method for the isolation of scales by differential centrifugation (P — pellet; S — supernatant).

separate the different scale types because there was a small yield of scales. Scale fraction A was collected from cells that had been lysed in distilled water (10 min) and further disrupted (5 min) in a Dawe sonicator Type 114B (frequency 20 kc $s^{-1}$; power output 50 W). A surfactant, Triton-×-100 was added to the cell slurry (1% v/v) to aid disruption during sonication. Scale fraction B contained scales that were 'free-floating' in the medium. The initial low gravity centrifugation step (100 × g for 5 min) was included to prevent the scales being stripped from cells during sedimentation. Scale fractions were repeatedly washed with distilled water and the purity of the final fractions was checked with the transmission electron microscope (TEM) by observing Au/Pd shadowed samples mounted on formvar-coated grids.

It is important to note that the algal cultures used were xenic (not bacteria-free). However, preparative techniques did not cause disruption of the bacteria in the cell pellet used to obtain fraction A scales. This was confirmed by viewing heavy metal shadowed preparations of the cell slurry in the TEM. Bacteria in scale fractions A and B were easily removed by differential centrifugation.

### Treatment of scales

*(a) General chemical nature of scales*

The chemical nature of scales was determined by subjecting them to a number of chemical and enzymatic treatments summarized in Table 1. A small suspension of scales was placed into 1 ml of the chemical solution and incubated for the required period. The effect of these treatments on scales was determined by viewing shadowed preparations in the TEM. Scales were also stained with alcian blue at pH 2,5 and pH 0,5 (Crayton 1982).

**Table 1** Enzymatic and chemical treatment of scale fractions

(i) 5% Ethylenediaminetetra-acetic acid ($Na_2$ EDTA) for 8 h at 20°C (pH 7,1).
(ii) Distilled water for 8 h at 100°C.
(iii) 0,1N HCl
(iv) 0,1N NaOH } 8 h at 20°C
(v) 1,0% Cellulase (Sigma; activity 0,5 – 1,0 unit per mg solid) for 12 h at 20°C; buffered to pH 5 using McIlvain's buffer (Hale 1958).
(vi) 1,0% Pectinase (Sigma; activity 1 unit per mg solid) for 12 h at 20°C; buffered to pH 4 using McIlvain's buffer.

*(b) Analysis of sugar residues in scale fractions*

Subfractions from scale fractions A and B were freeze-dried and then hydrolysed *in vacuo* in 2N $H_2SO_4$ at 100°C for 1 h. The hydrolysates were cooled and neutralized with $BaCO_3$ using methyl red as an indicator. The $BaSO_4$ precipitate was removed by centrifugation and the supernatant was taken to dryness in a vacuum evaporator. Sugar residues were dissolved in 2 ml 10% aqueous isopropanol before being spotted (100 – 250 µl) onto thin layer chromatography plates (Merck silica gel 60 $F_{254}$ precoated plates).

Reference sugars (L-rhamnose, D-glucose, D-galactose, D-ribose, D-xylose, L-arabinose, D-fructose and D-mannose), made up as 1% solutions in 10% isopropanol, were spotted (10 µl) alongside the scale hydrolysates. The plates were developed by ascending chromatography in n-butanol:acetic acid:ether:water (9:6:3:1, Harborne 1973). When the solvent front had moved to 16 cm (± 4 h) the chromatograms were air-dried and sprayed with one of two reagents; (i) 0,2% naphthoresorcinol in n-butanol containing 10% (v/v) phosphoric acid (Harborne 1973), (ii) 0,1% naphthoresorcinol in ethanol containing 20% (v/v) 2N HCl (Bell 1955).

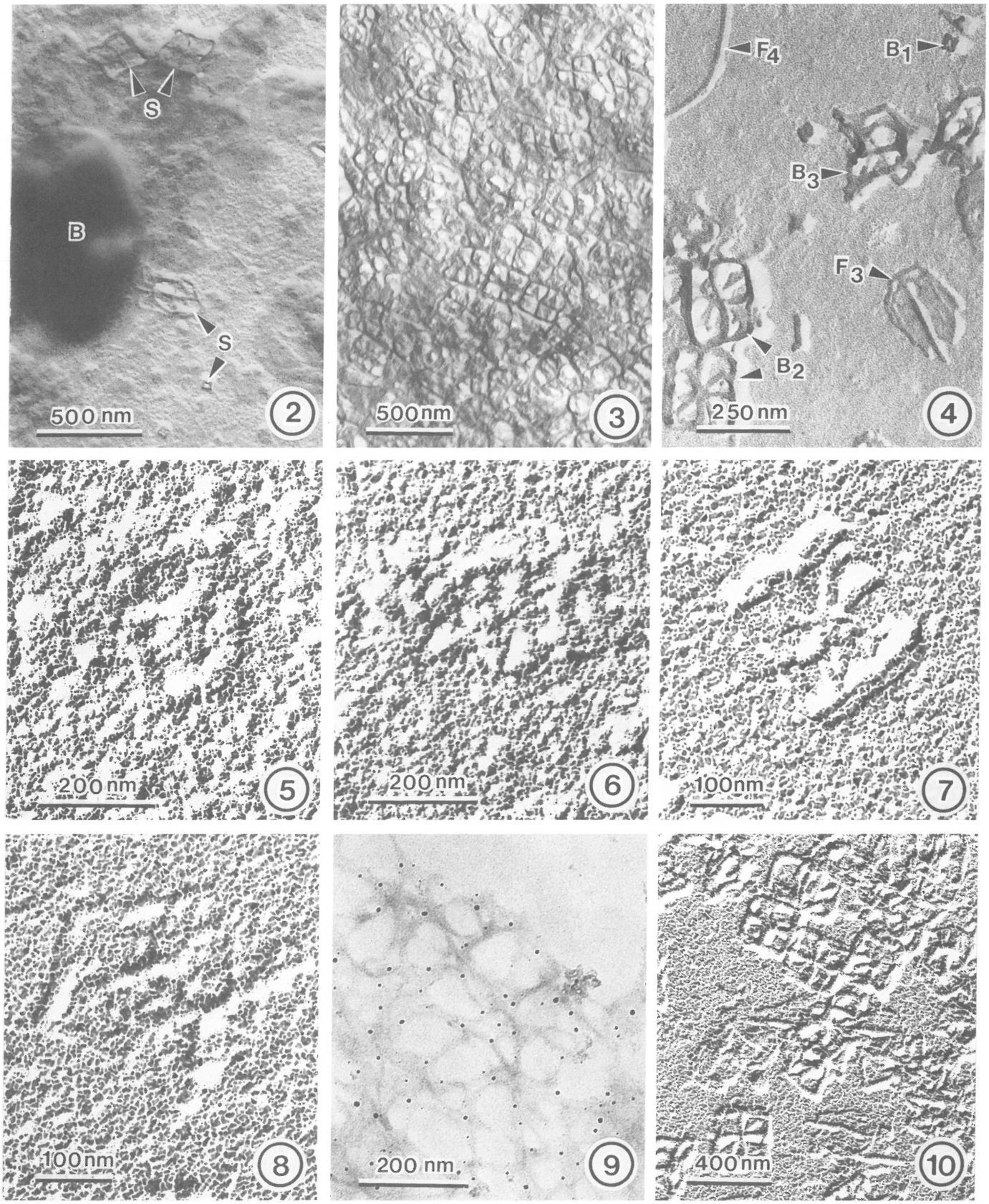
Coloured sugar spots were differentiated after heating the plates to 100°C (10 min) and 75°C (5 min), respectively.

*(c) Analysis of amino acid residues in scale fractions*

Scale fractions were freeze-dried and hydrolysed *in vacuo* in 6N HCl for 24 h at 110°C. The hydrolysates were analysed on a Beckman Model 119 amino acid analyser (column packed with Beckman AA15 cation exchange resin).

### Treatment of cyst wall

Living or fixed cysts were subjected to the following histo-




**Figures 2 – 10** **(2)** Cell slurry showing an intact bacterium (B) and scales (S) amongst the cell debris. **(3)** Dense pellet of scales obtained by differential centrifugation. **(4)** A diluted sample from scale fraction B showing individual scale types ($B_3$ — distal body scale; $B_2$ — intermediate body scale; $B_1$ — proximal body scale; $F_3$ — limuloid flagellar scale; $F_4$ — hair scale). **(5)** A degraded scale after treatment with EDTA. **(6)** A pectinase treated scale showing advanced degradation. **(7 & 8)** Scales treated with HCl showing partial degradation. **(9)** Scales showing distortion after hot water treatment. **(10)** Scales unaffected after treatment with cellulase.

chemical tests: PAS reaction for carbohydrates (Chayen *et al.* 1969); KI-$I_2$/$H_2SO_4$ test for cellulose (Gurr 1965); alcian blue tests (pH 2,5 and pH 0,5) for acidic (sulphated) polysaccharides (Crayton 1982); and Sudan black B test for lipid (Chayen *et al.* 1969). Cysts were also subjected to acetolysis to test for sporopollenin (Atkinson *et al.* 1972). Some cysts were embedded in Spurr's resin using the method described by Pienaar & Aken (1985). The elemental composition of the

cyst wall was determined (in 5 μm sections) by energy dispersive X-ray (EDX) analysis in a Jeol 35 scanning electron microscope fitted with a Kevex X-ray detector.

## Results

The fact that xenic algal cultures had to be used in this study caused some concern as there was always the possibility that disrupted bacterial cells could contaminate the scale fractions. Fortunately, though, treatment with distilled water followed by mild sonication disrupted the algal cells but had no effect on the bacterial contaminants. The bacteria were then easily removed from the scales by differential centrifugation. An intact bacterium is visible amongst algal debris after sonication in Figure 2. After repeated washings using differential centrifugation, a clean pellet of pure scales was obtained (Figure 3). A diluted sample from fraction A scales is shown in Figure 4. All scale types were represented in both scale fractions but there were proportionally fewer underlayer scales because some of these were lost with each successive wash.

### Chemical composition of scales

Scales treated with the chelating compound EDTA and the enzyme pectinase were rendered soluble during the incubation period. Only a few scales in advanced stages of degradation were observed (Figures 5 & 6, respectively). Scales were apparently degraded with NaOH as no scales were seen on grids after this treatment. Scales treated with HCl were degraded to varying extents (Figures 7 & 8). Scales that had been treated with hot water were distorted, indicating that some extraction had taken place (Figure 9). Cellulase did not affect the scales (Figure 10). Untreated scale pellets were readily stained with alcian blue at pH 2,5 and pH 0,5 indicating that the scales are composed of a sulphated acidic polysaccharide.

Sugar residues detected in scale fractions A and B are shown on the chromatogram in Figure 11. These sugars, which were

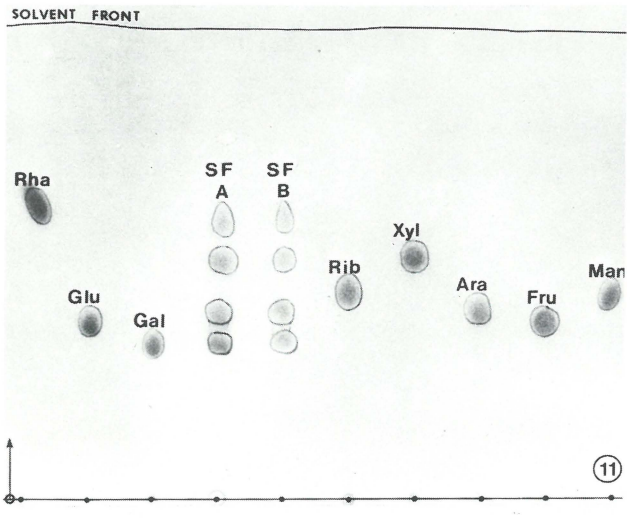



**Figure 11** A chromatogram showing the sugar residues detected in scale fractions A (SF A) and B (SF B). Rha — rhamnose, Glu — glucose, Rib — ribose, Xyl — xylose, Ara — arabinose, Fru — fructose, Man — mannose and Gal — galactose.

identified by their colour reaction with naphthoresorcinol (Harborne 1973) and co-chromatography with reference sugars, are believed to be galactose, arabinose, xylose and rhamnose. Based on the intensity of the spots galactose was

**Table 2** RF values (× 100) of reference sugars and sugars in scale hydrolysates

| | Reference sugars | Scale Fraction A | Scale Fraction B | Colour with naphthoresorcinol/ butanol/$H_3PO_4$ |
|---|---|---|---|---|
| Rhamnose | 62 | 59 | 59 | blue |
| Glucose | 37 | | | blue |
| Galactose | 32 | 33 | 33 | blue |
| Ribose | 44 | | | green |
| Xylose | 51 | 51 | 51 | green |
| Arabinose | 39 | 40 | 40 | green |
| Fructose | 37 | | | brown |
| Mannose | 44 | | | blue |

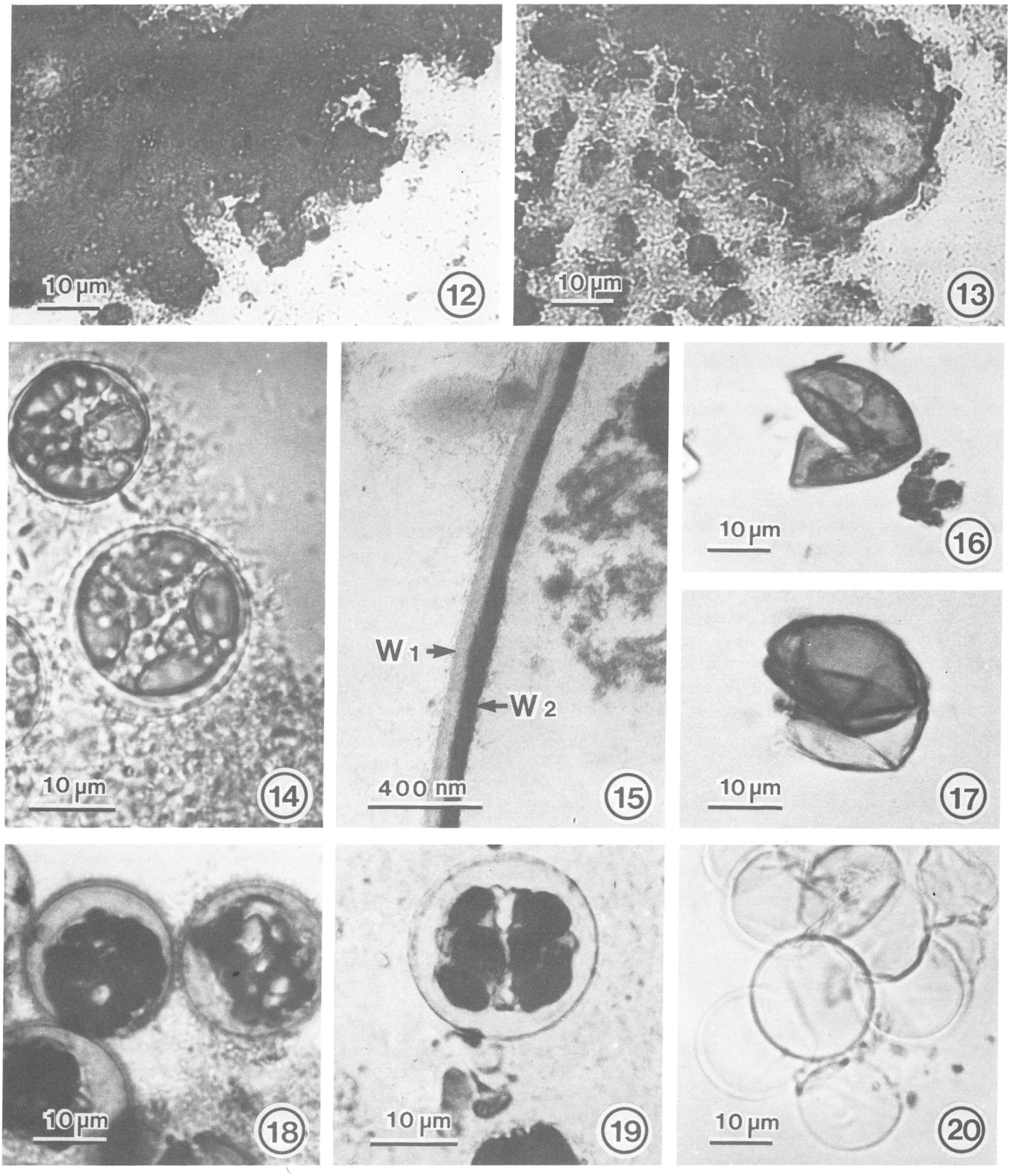
the predominant sugar. Arabinose and xylose were present in higher concentrations than was rhamnose. Although the rhamnose component in the hydrolysates appeared to run more slowly than the reference sugar it does co-chromatograph with the reference sugar when the hydrolysate is not as heavily loaded. The Rf values of the reference sugars and sugars in the scale hydrolysates are given in Table 2. The spray reagent naphthoresorcinol/butanol/$H_3PO_4$ proved to be very useful in differentiating the different sugars which were not distinctly separated. After heating, hexoses and pentoses were revealed as blue and green spots respectively. Fructose appeared as a brown spot. In the chromatographed hydrolysates the second most polar spot could be glucose, fructose or arabinose. The distinct green colour of this spot indicated that it contained predominantly arabinose although lower concentrations of the other two sugars could be present.

The paler spots in scale fraction B signified a lower concentration of sugars in this hydrolysate. This was expected because only 1,5 mg (dry weight) of scales were collected in scale fraction B compared with 7,3 mg in scale fraction A.

The second spray reagent used (Bell 1955) was chosen because it supposedly reveals uronic acids when sprayed chromatograms are dried at room temperature. The digestion of scales with pectinase suggested that they contained a polygalacturonide component and it was therefore surprising that no uronic acid was detected in chromatograms sprayed with this reagent. It is important to mention here that a gelatinous

**Table 3** Amino acid residues in the scale hydrolysates (expressed as percentages of sample dry weight)

| Amino acid residues | Scale Fraction A | Scale Fraction B |
|---|---|---|
| Aspartic acid | 0,52 | 0,40 |
| Threonine | 0,27 | 0,19 |
| Serine | 0,24 | 0,17 |
| Glutamic acid | 0,50 | 0,33 |
| Proline | 0,19 | 0,10 |
| Glycine | 0,22 | 0,16 |
| Alanine | 0,28 | 0,18 |
| Valine | 0,38 | 0,27 |
| Methionine | 0,10 | 0,05 |
| Isoleucine | 0,19 | 0,14 |
| Leucine | 0,38 | 0,21 |
| Tyrosine | 0,19 | 0,10 |
| Phenylalanine | 0,27 | 0,16 |
| Histidine | 0,08 | 0,02 |
| Lysine | 0,23 | 0,09 |
| Arginine | 0,22 | 0,12 |
| Estimated protein content (sum of residues) | 4,50% | 3,19% |




**Figures 12 – 20** **(12 & 13)** Acid resistant scale residue stained with alcian blue at pH 2,5 (12) and pH 0,5 (13). **(14)** Two mature cysts of *P. pseudoparkeae*. **(15)** A section showing the bilayered nature of the cyst wall. The outer layer ($W_1$) is electron translucent while the inner layer ($W_2$) is osmiophilic and electron dense. **(16)** The cyst stained with alcian blue at pH 2,5 and **(17)** pH 0,5. **(18)** Cysts stained with Sudan black B. The cyst wall and lipid globules are stained while starch is unaffected. **(19)** A PAS treated cyst. Only the starch grains are reactive. **(20)** Cyst walls after acetolysis treatment (10 min).

residue remained after the scales were hydrolysed in 2N $H_2SO_4$. This residue stained readily with alcian blue at pH 2,5 and pH 0,5 (Figures 12 & 13, respectively) but was not digested with pectinase. Further treatment in 5N $H_2SO_4$ (100°C) did not obviously affect the residue although a trace of fructose only was detected in chromatographed hydrolysates.

The amino acid residues detected in the scale hydrolysates are given in Table 3. Aspartic acid and glutamic acid predominated in both scale fractions. Since non-protein amino acids were absent, it is assumed that the amino acid residues were derived from protein in the scales. If so, then scale fractions A and B contained 4,5% and 3,19% protein, respectively. In the absence of replicate analyses it is impossible to know if this difference is significant.

## Cyst wall histochemistry

Encysted cells of *P. pseudoparkeae* (Figure 14) possess a thick, bilayered cell wall (Figure 15). The outer amorphous layer is electron translucent while the inner fibrillar layer is osmiophilic and electron dense. To obtain any satisfactory staining of the intact cyst wall (in living or dead cells) long exposures to the stain reagents were required. However, where the cyst wall was ruptured, either physically or during excystment, the stain was taken up rapidly. In many preparations the fortuitous delamination of the cyst wall (see Figure 17) revealed that only the inner component of the wall reacted with the stains used.

The inner layer of the cyst wall bound alcian blue stain at pH 2,5 and pH 0,5 (Figures 16 & 17, respectively). It also stained with Sudan black B (Figure 18). Cell walls treated with $KI\text{-}I_2/H_2SO_4$ did not swell nor did they assume the blue coloration typical of cellulosic walls. Both components of the cyst wall did, however, stain a pale straw colour with Gram's iodine before irrigation with $H_2SO_4$.

The most surprising observation made was that the cyst wall was PAS-negative. Starch grains within the cyst, however, showed a positive reaction to the test (Figure 19). The outer component of the cyst wall was extremely resistant and withstood acetolysis for 10 min (Figure 20).

The elemental composition of the cyst wall, determined by EDX analysis, is shown in Figure 21. The small peaks for

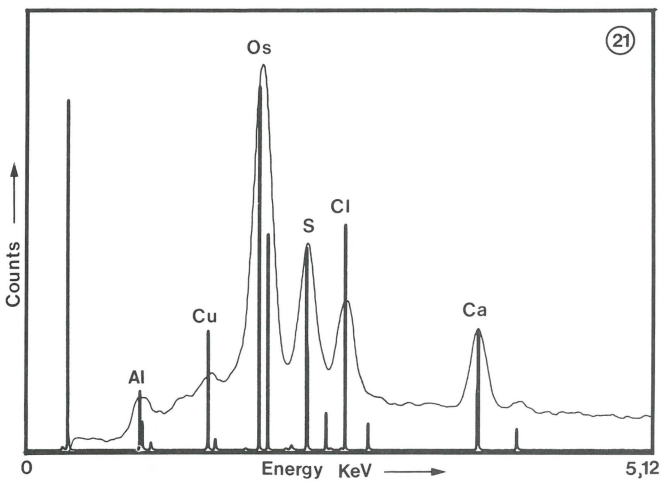
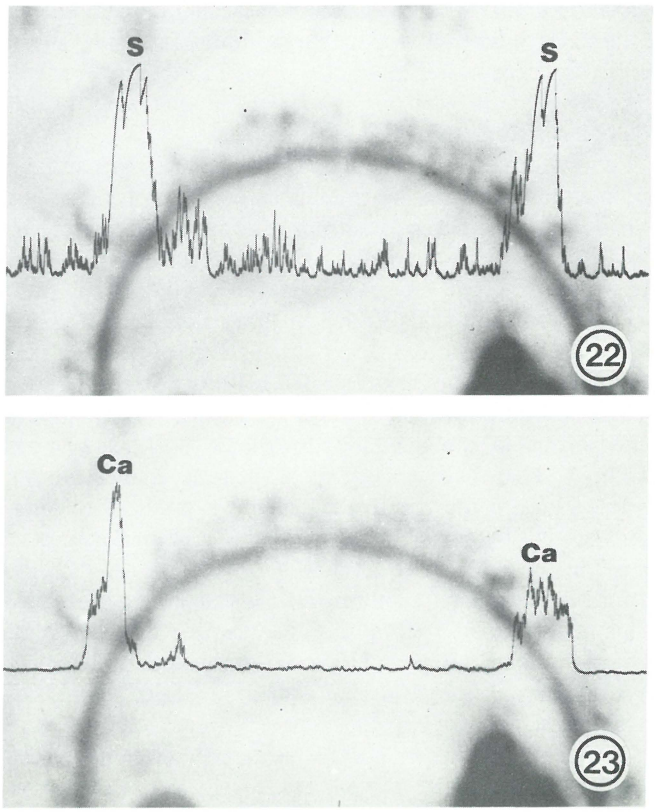



**Figure 21** A spectrum of the elements detected in the cyst wall using the EDX analysis. Al — aluminium, Cu — copper, Os — osmium, S — sulphur, Cl — chlorine and Ca — calcium.

aluminium and copper represent contamination from the copper viewing grids and can be ignored. Since the cells were post-fixed in osmium tetroxide, and because the inner layer of the cyst wall is known to be osmiophilic (refer to Figure 15), the osmium peak was expected and can be ignored because the element is not present in cells fixed with glutaraldehyde only. The peak for chlorine can also be disregarded because this element is present in Spurr's resin. Most important are the peaks for sulphur and calcium. Scans made across the cyst in the sulphur and calcium windows show that these elements are concentrated in the wall (Figures 22 & 23, respectively).




**Figures 22 – 23** Line scans made across the cyst showing that sulphur (22) and calcium (23) are concentrated in the cyst wall.

## Discussion

The scales of *P. pseudoparkeae* are not cellulosic in composition because they were not digested with the enzyme cellulase. Rather they appear to be pectinaceous in composition having many of the characteristics of plant pectins. Evidence supporting this interpretation is presented below.

The digestion of scales with pectinase suggests that they are composed, at least in part, of a polygalacturonide because this enzyme (Poly-{1,4-α-D-galacturonide}-glycanohydrolase) hydrolyses α-1,4 linkages between D-galacturonic acid residues in pectins (Aspinall 1970). Furthermore, the degradation of scales in EDTA indicates that the polygalacturonic acid polymers are probably cross-linked with divalent cations. Pectins characteristically contain divalent cations, usually calcium, which form complexes with the carboxyl groups on galacturonic acid residues. These cations act as transverse linkages joining adjacent polymers of galacturonic acid (Smith 1977) and in the presence of a chelating agent such as EDTA they are removed thus breaking the ionic bridges between chains and rendering the pectin soluble (Hall *et al.* 1974). This may explain the degradation of scales treated with EDTA.

Histochemical support for the presence of an acidic component in the scales is seen in their ability to bind alcian blue at pH 2,5 and pH 0,5. Their ability to bind the stain at pH 0,5 is particularly interesting because it indicates that the polysaccharide is sulphated. At this very low pH, carboxyl groups do not dissociate so that alcian blue complexes exclusively with sulphate groups (Parker & Diboll 1966; Aminoff *et al.* 1970; Crayton 1982). Sulphate groups are presumed to be associated with the polysaccharide rather than with the protein component of the scales. Sulphate esters of proteins are seldom free to react, and if they do, no appreciable colouration is produced under histochemical conditions (Aminoff *et al.* 1970). The four neutral sugars detected in the scale hydrolysates, viz. galactose, arabinose, xylose and rhamnose, are common constituents of pectic substances (Cook & Stoddart 1973). Although it is not known how these

sugars are linked with the acidic polysaccharide component of the scales, it is worth noting that rhamnose is the only sugar known to occur in the internal chains of polygalacturonans while all other neutral sugars are encountered only in the external chains (Aspinall 1973, 1980).

The absence of uronic acid on the chromatograms and the presence of a gelatinous residue in the hydrolysis vessels indicated that the polygalacturonic acid withstood hydrolysis. The difficulty attending hydrolysis of uronic acids has often been reported (Bell 1955; O'Colla 1962; Albersheim 1965; Percival & McDowell 1967; Sharon 1975). Because this residue bound alcian blue at pH 0,5, it is tempting to speculate that the sulphate groups in unhydrolysed scales are associated with this acidic component. It is possible, though, that sulphuric acid contributed sulphate during hydrolysis. That the polymers in the residue had undergone structural change during hydrolysis was confirmed by the inability of pectinase to digest this material. Percival & McDowell (1967), in their treatment of sulphated acidic polysaccharides, have shown that the mucilages of *Ulva* (L.) Thuret and *Enteromorpha* Link have sulphate esters on rhamnose. The possible association of sulphate with rhamnose and the possible presence of this sugar in the internal galacturonan chains in scales should be investigated.

As our observations on the scale carbohydrate have been purely qualitative, the concentrations of sugars in the scale polysaccharide need to be determined in future studies.

The amino acid residues detected in scale hydrolysates are believed to originate from protein in the scales. Although not substantiated here, Domozych *et al.* (1980) and Melkonian *et al.* (1983) have reported that protein is present in prasinophyte scales. Since both scale fractions A and B contained comparable amounts of protein (4,5% and 3,19%, respectively), with similar proportions of different amino acid residues, it is probable that this is a close approximation of the protein content of scales. It is unlikely that the protein detected in scale fraction B could be attributed to protein contamination by absorption because this fraction contained 'free-floating' scales that were not associated with a cell slurry.

The nature of the protein/carbohydrate complex in scales needs to be investigated because a covalent linkage between the two components would indicate that the scales are composed of glycoprotein. The scales are in all probability glycoproteinaceous but we have refrained from using the term because we have no evidence to show that the protein/carbohydrate components are covalently linked. Scale hydrolysates were not examined for hydroxyproline. Its presence or absence in scales should be established because of its potential taxonomic value (Lewin 1974).

The sugar residues in scales are remarkably similar to those detected in thecal hydrolysates of *Tetraselmis* (Lewin 1958; Gooday 1971). Only xylose and a trace of fructose are additional sugars in scales. These results provide chemical evidence that the theca of *Tetraselmis* and the periplast of *Pyramimonas* may be homologous structures. Melkonian *et al.* (1983) have recently shown that the flagellar scales of *Tetraselmis* are glycoproteinaceous. If the scales of *Pyramimonas* are also composed of glycoprotein then it is reasonable to speculate that the cell walls of those chlorophycean algae containing glycoprotein (e.g. *Chlamydomonas,* Roberts *et al.* 1972) may have evolved, as Mattox & Stewart (1977) have proposed, by the fusion of scales in a prasinophyte like *Pyramimonas.* The composition of scales in other prasinophytes should be investigated for comparative purposes. The abundance of hydroxyproline in the cell wall of *Chlamydomonas* emphasizes the need to determine if this amino acid is present in prasinophyte scales.

This basic knowledge of scale composition allows a few comments to be made on the possible mechanisms underlying the external assembly of scales within the scale-boundary, and the possible functions of scales. Scales released from the cell always occupy precise positions in the scale-boundary and often show a definite orientation; this is most noticeable in limuloid flagellar scales for example. The uronic acid residues and sulphate groups in the scale polysaccharide undoubtedly impart a high negative charge to scales. We propose two mechanisms that could explain how different scale types arrive at their respective positions in the scale-boundary.

The first is that different scale types may possess different charge densities, resulting in a shuffling of scales in the scale-boundary according to a charge density gradient from underlayer scales to the outermost scale layer. Ion exchange chromatography or electrophoresis could be used to determine charge density differences between different scale types. Moreover, these techniques may be useful in separating different scale types for chemical characterization. The second mechanism, which could be working in conjunction with the first, is that scales may possess characteristic patterns of surface charges, providing a 'lock and key' mechanism for the orientation and positioning of scales. In this respect the role of protein in the external assembly of the scale-boundary should not be overlooked. It may be no coincidence that the predominant amino acids, aspartic and glutamic acid, are ionized over wide pH ranges so that reactive groups in these residues could be available for ionic bonding.

Tannic acid, known for its protein binding capacity (Swain 1965; Schanderl 1970), has been shown to stabilize scales in the scale-boundary (Melkonian *et al.* 1981). This would indicate that protein is perhaps playing a role securing scales, especially so in linking the underlayer scales to the plasmalemma in the 'basal scale plate' region.

Charged scales may be secured in the scale-boundary by ionic bonds involving divalent cations. Divalent cations, already shown here to play a role in maintaining scale structure, may also be involved in ionic cross-linkages between scales in the scale-boundary. Melkonian (1982b) has shown that the presence of rod scales on the flagella of *Tetraselmis* is dependent on the concentration of calcium ions in the culture medium. He suggested that loss of rod scales during glutaraldehyde fixation may be attributed to the interference of glutaraldehyde with calcium-binding sites which could link adjacent rod scales to one another and to the underlayer scales. He also reported that the underlayer scales were not as sensitive as rod scales to a decrease in calcium ion concentration and fixation procedures, and were not as easily lost. This suggests that in *Tetraselmis* the different scale types have different chemical compositions. This work should be extended to the genus *Pyramimonas* and emphasizes the need to determine the chemical make-up of individual scale types.

While not much is known about the functional significance of scales in green algae, Melkonian *et al.* (1981) have suggested that they may have a protective function and may play a role in divalent cation metabolism, either providing an extracellular store of calcium or enabling cells to survive in a calcium rich environment. Building on the latter, we propose that scales, by virtue of their polyanionic nature, contribute to the maintenance of water and salt balance in cells. Acidic polysaccharides, rich in carboxyl and sulphate groups, are known to have ion exchange properties that may be important in regulating the water and salt balance of cells (Sharon 1975),

especially in organisms living in a saline medium (Percival & McDowell 1967).

### The cyst wall

The staining properties of the inner layer of the cyst wall indicate that it is similar in composition to scales. It binds alcian blue at pH 2,5 and pH 0,5 and therefore contains a sulphated acidic polysaccharide. The presence of sulphur in the cyst wall was confirmed by EDX analysis. Calcium in the cyst wall (which may also be the divalent cation present in scales) could be functioning to form cross-linkages between acidic polysaccharide chains.

The inner layer of the cyst wall contains lipoidal material which stained with Sudan black. This was not unexpected as this layer is known to be osmiophilic.

The most surprising histochemical result obtained was that the cyst wall was PAS-negative: The PAS reaction is a standard histochemical test for the localization of polysaccharide material (Gurr 1965; Pearse 1968; Chayen *et al.* 1969). Because the inner layer of the cyst wall is proposed to contain polysaccharide material this result was unexpected. One explanation for our observations is obtained from Aminoff *et al.* (1970) where they report that polysulphates may or may not be PAS-positive depending on the degree of sulphation. The preponderance of sulphur in the elemental spectrum of the cyst wall suggests that the high concentration of sulphate groups present may have given a PAS-negative result. Pearse (1968) has also reported that acid mucopolysaccharides are PAS-negative.

The material comprising the outer layer of the cyst wall is considered to be sporopollenin because this compound is 'the only known organic constituent of plant cell walls that withstands acetolysis for more than a few minutes . . . ' (Atkinson *et al.* 1972). This, however, needs to be confirmed using infra-red spectroscopy. The presence of sporopollenin in the cyst wall of *Pyramimonas* has not been reported before. This compound is probably more widely distributed in the Prasinophyceae than is generally recognized. Furthermore, its presence in the fossil prasinophyte *Tasmanites* Newton (Brooks 1971) indicates that the compound has had a long history in the class.

The cyst wall of *P. pseudoparkeae* is strikingly similar to the cell wall of the phycoma of *Halosphaera* Schmitz which also has an outer sporopollenin component and inner pectic component (Parke & Den Hartog-Adams 1965). This, in addition to the similarity in structure of the motile cells of the two genera (Parke & Adams 1961; Manton *et al.* 1963; Pennick 1977), provides further evidence that they are phylogenetically closely related. *Pterosperma* Pouchet and *Pachysphaera* Ostenfeld also have bilayered cell walls of sporopollenin and pectic material (Wall 1962; Boalch & Parke 1971; Parke *et al.* 1978).

Sporopollenin is not common in the green algae but has been found in *Pediastrum* Meyen (Brooks & Shaw 1971), *Chlorella* Beijerinck (Atkinson *et al.* 1972), *Scenedesmus* Meyen (Staehelin & Pickett-Heaps 1975) and *Trebouxia* de Puymaly (König & Peveling 1980) in the Chlorophyceae and in *Chara* Vaillant in the Charophyceae (Brooks & Shaw 1971).

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